RESEARCH PAPER

First report of an endophyte (*Diaporthe phaseolorum* var. *sojae*) from *Kandelia candel*

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Abstract: Mangrove endophytic fungus 1893 was isolated from *Kandelia candel* from an estuarine mangrove on the South China Sea Coast. Two new lactones 1893A and 1893B, together with other known compounds, have been isolated from its fermentation broth. To classify the endophyte correctly for further industrial application, a combination of morphological and molecular techniques was used to approach its identity. The endophyte was compared with similar species having trichogynes or trichogyne-like hyphae which apparently fused with antheridium-like hyphae, and perithecia initials developing from an ascogonial coil surrounded by enveloping hyphae in early developmental stages on pure culture. Further morphological characteristics on host and non-host were used for comparison with similar species when the endophyte was cultivated on leaves of *Kandelia candel* and *Mangifera indica*, respectively, which resulted in classifying the endophyte as a *Phomopsis* species. The ITS sequence of rDNA was used to infer its phylogenetic relationships with *Phomopsis* species that resembled the strain in morphology or ecology. Finally, the endophyte was identified as *Diaporthe phaseolorum* var. *sojae* based on morphological and molecular evidence. Our study is a first report of *Diaporthe phaseolorum* var. *sojae* isolated from mangrove *Kandelia candel*.

Key words: *Phomopsis*; mangrove endophyte; morphology; phylogeny

Introduction

Mangrove forests are mostly distributed in tropical and subtropical regions (Gilbert & Mejía-Chang 2002), and several mangrove species are a valuable source of useful metabolites for medicinal usage (Kathiresan & Bingham 2001). Some of the potency of mangrove plants may be due to mutualistic fungal endophytes associated with host plants (Selosse et al. 2004; Suryanarayanan et al. 1998). In fact fungi from mangroves are the second largest group among the marine fungi (Sheadrer et al. 2007). The practical applications of mangrove endophytic fungi are manifold, as potential bio-control agents, sources of novel metabolites for therapeutics, plant protection, and other industrial

applications (Wu et al. 2005; Yuan et al. 2005). Our group has recently undertaken several studies on mangrove endophytic fungi from South China Sea coast and has isolated various bioactive metabolites (Lin et al. 2001; Zeng et al. 2006).

In the course of our search for novel bioactive compounds from marine fungi, two new lactones 1893A, 1893B and several other known compounds (Chen et al. 2003; Chen et al. 2006) have been isolated from the fermentation broth of the endophytic fungus No. 1893, which was collected from *Kandelia candel* in an estuarine mangrove on the South China Sea Coast. Preliminary morphological examination revealed that the strain was a mycelia sterilia. No asci, ascospores or conidia were produced either on PDA or other artificial media, in 60-d colonies, only sub-ovoid, light-yellow sclerotia are present, becoming pale brown as they mature. Therefore, it is not possible to name the species, which results in hindering the exploration of mangrove endophytes for industrial application.

Recently, the nucleotide sequences of the internal transcribed spacer (ITS), large subunit (LSU) nuclear ribosomal DNA, EF-1α gene and other ribosomal DNA regions as well as RAPD analysis have been successfully used to determine the phylogenetic relationships of species in many diverse genera (Castlebury et al. 2002; Castlebury et al. 2003; Chi et al. 2007; Farr et al. 2002; Fernández & Hanlin 1996; Promputtha et al. 2007; Schilder et al. 2005), especially for *Phomopsis* species identification, as old *Phomopsis* cultures would lost the ability to produce pycnidia, which is important for providing taxonomically useful information (Farr et al 1999; Uecker 1989). Thus it would be necessary to use nucleotide sequences of ribosomal DNA regions if one want to infer the relationships of mycelia sterialia with

other fungi, (Guo et al. 2000; Mucciarelli et al. 2002; Zhang et al. 1997). The aim of this paper is to: (1) induce the endophyte to produce reproductive structures for identification; (2) compared the endophyte with other similar species and inferred its phylogenetic relationships from ITS region by maximum Likelihood, Neighbor-joining and Bayesian analysis with special attention to fungi that resemble the strain in morphology or ecology.

Materials and Methods

Fungal Material

The mangrove endophytic fungus 1893 was isolated from the South China Sea coast by Professor Vrijmoed LLP, City University of Hong Kong, China. The strain was grown on potato-dextrose-agar slant for fortnight at 25ºC, maintained at 4ºC and sub-cultured every three month. Vouchers of the fungus have been deposited in the College of Life Science and School of Chemistry and Chemical Engineering, Sun Yat-sen University, and City University of Hong Kong, China.

Morphological examination

Mangrove endophytic fungus 1893 was grown in the dark at 25ºC. Media used were potato-dextrose agar (PDA), glucose-peptone-yeast-extract agar (GPY: glucose 20 g/L, peptone 5g/L, yeast extract 5g/L), and 2% malt-extract agar (MEA). Growth rates and colony diameters of cultures incubated in the dark were measured on PDA. Colony morphology and growth of the spore formation were checked on PDA, GPY and MEA. Sterile cover slides were placed close to selected colonies, and the fungal cells were allowed to grow onto the slides. Slides with attached fungal cells were mounted with water. The endophyte was also induced to form reproductive structures by growing it on autoclaved leaves of *Kandelia candel* and *Mangifera indica*, respectively, on PDA at room temperature. Images were captured on a Nikon Eclipse TE200-U microscope with bright field and epifluorescence optics and Nikon digital camera DXM1200F using SPOT software or on an Olympus Szx7 research stereo microscope with software Auto-Montage Pro. Measurements in the description were given as (i) mean$\pm$SD (ii) (n1－)n4－n3－n5(－n2),with n1 = minimum value observed; n2 = maximum value observed; n3 = arithmetical means; n4/n5 = first/third quartile.

Phylogeny

A hyphal tip was obtained from a fresh culture and was grown on PDA in the dark at 25ºC for 7d. Fresh fungal mycelia (c. 50 mg) were scraped from the surface of the agar plate and ground in liquid nitrogen. Fungal genomic DNA was prepared using GALEN mini Kit (Catalogue no.69104, Galen biopharm international co. LTD, Beijing, China) according to the manufacturer's protocols. ITS sequence was bound by primers ITS1 and ITS4 (http://www.biology.duke.edu/fungi/mycolab/primers.htm). PCR reaction mixes contained 2.5 μL 10×PCR buffer, 5 μM dNTP,

12.5 pM of each PCR primer, and 5 μL DNA in 25 μL. The amplification program included 40 cycles of 94ºC for 30 s, 50ºC for 30 s, and 72ºC for 1 min. To obtain readable sequence data of the ITS region, the PCR products were cloned into the pMD18-T vector (Takara) and sequenced by the dideoxynucleotide method. This procedure was repeated twice and the two sequences generated were the same, one of them was submitted to GenBank (accession number: EU117221). Other sequences used in this study were obtained from the GenBank.

Phylogenetic analysis

Sequences were aligned with ClustalX (Thompson et al. 1997) and adjusted by eye in the data editor of PAUP* 4.0b10 (Swofford 2002). All data sets were analyzed in PAUP* 4.0b10 and MrBayes 3.1 (Huelsenbeck & Ronquist 2001), with gaps treated as missing data. Neighbor joining trees were using the Kimura two-parameter distance as implemented in PAUP* 4.0b10 with 1000 replications.

  Likelihood analysis was performed using a model and model parameters estimated with Modeltest 3.7 (Posada & Crandall 1998). A likelihood command block was copied from Modeltest3.7, which configures PAUP* to search for a maximum likelihood (ML) tree under the GTR+I+G model with 10 heuristic search replicates, each with a random taxon addition sequence, MAXTREES set to autoincrease, and TBR branch swapping (Wang et al. 2005). The rate matrix, assumed nucleotide frequencies, assumed proportion of invariable sites and gamma distribution were estimated by AIC in modeltest3.7. In addition to the maximum likelihood analysis, Bayesian posterior probabilities were computed using the Markov chain Monte Carlo method (MCMC) in MrBayes 3.1 by running with 1000000 generations and sampled every 100 generations using the program default priors on model parameters. Posterior nodal probabilities were summarized by generating a majority rule consensus tree by using PAUP.

Results

Morphological examination

Colonies grown on PDA, moderately fast growing, attaining a diameter of 3.9 mm after 5d and 9 mm after 14d at 25ºC; producing white mycelia with occasional greenish-yellow surface areas turning light brown with age (Fig. 1: A-B); aerial hypae often abundant, usually fasciculate to form mycelial strands on pure culture or when in contacted with inert and hard objects, i.e., a cover glass lined on the agar medium. On these surfaces, simple or double hyphal rings, apparently fused with antheridium-like hyphae and perithecia initials developing from an ascogonial coil surrounding by enveloping hyphae in early developmental stages (Fig. 1: C, F, H-J) , like the perithecial development in representative Pyrenomycetes (Luttrell & Huang, 1982; Maiello, 1978; Sanni, 1982), (19.20－)23.64－29.65－35.63(－49.50) μm, have been observed in 30d colonies; sub-ovoid, light-yellow sclerotia (Fig. 1: K-L), diameter 49.9$\pm$

15.47 μm, were present, became pale brown as they mature. No fungal spores were observed, either on MEA medium or on PDA. Culturing the endophyte on all pure media at 25ºC, 18ºC and 4ºC in dark or treatment with UV lights at 25ºC was ineffective for the development of spore formation.
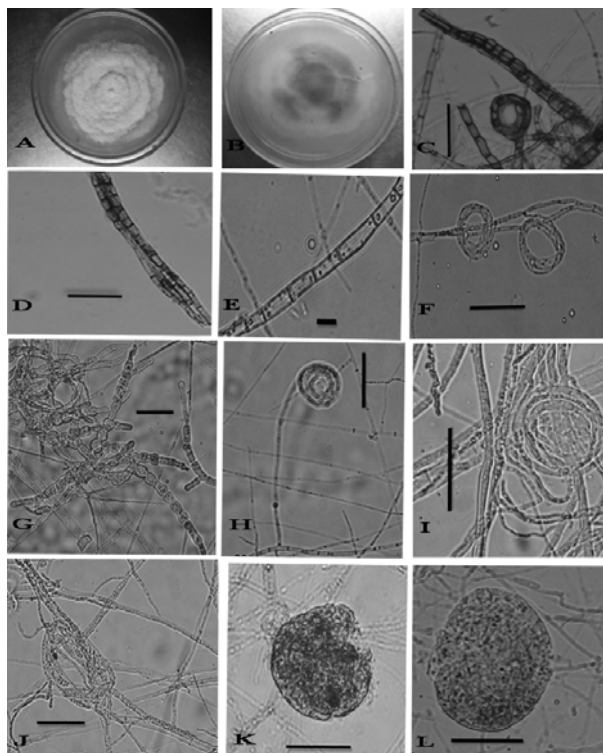

Fig. 1 Morphological characteristics of mangrove endophytic 1893 on pure culture. A-B: colonies of the endophyte on PDA grown on 9-diameter Petri dishes after 7d; C, F, H-J: trichogynes or trichogyne-like hyphae which apparently fused with antheridium-like hyphae and perithecia initials developing from an ascogonial coil surrounding by enveloping hyphae in different developmental stages(C, cultivated on MEA; F, H, cultivated on PDA; I-G, cultivated on GPY); D: hyphal fusion after 30d on PDA; E: Hyphae with oil drops after 30d on GPY; G: irregular hyphae, like chlamydospore; K, L: crushed and uncrushed sclerotium. Bars C-D, K, and L: 40μm; F, I, J: 50 μm; H, G: 30 μm; E: 10 μm.

Finally, pycnidia were detected on leaves of *Kandelia candel* and *Mangifera indica* on PDA plates after cultivation for 60 days, and were variable in cultural characteristics: (1) grown on leaves of *Kandelia candel*, pycnidia (Fig. 2), lacking necks, solitary, and small or aggregated in large conidiomata, containing exudates only with ß-conidia, hooked, hyaline, aseptate, width (0.8－)1.2－1.39－1.57(－1.8) μm, length (19.31－)21.35－23.40－24.77 (－31.13) μm; (2) grown on leaves of *Mangifera indica*, stomata with long-necked pycnidia (Figure 3), length 384.3±13.0 μm, sometimes branched, length 448.1±43.9 μm after 60 days; some of them containing exudates only with α-conidia, unicellular, ovoid to oblong-fusoid, hyaline, sometimes 2-guttulate, width (1.10－)1.54－1.83－2.20(－2.99) μm, length (3.10－) 4.62－5.23－5.97(－7.78) μm. Conidiophores length (13.11－)17.63－20.58－23.05(－29.60) μm. Perithecia were not observed. As it corresponded well with the species description of the genus

Phomopsis, the endophyte was identified as *Phomopsis* sp. (Tucker 1935; Neven et al. 1997).
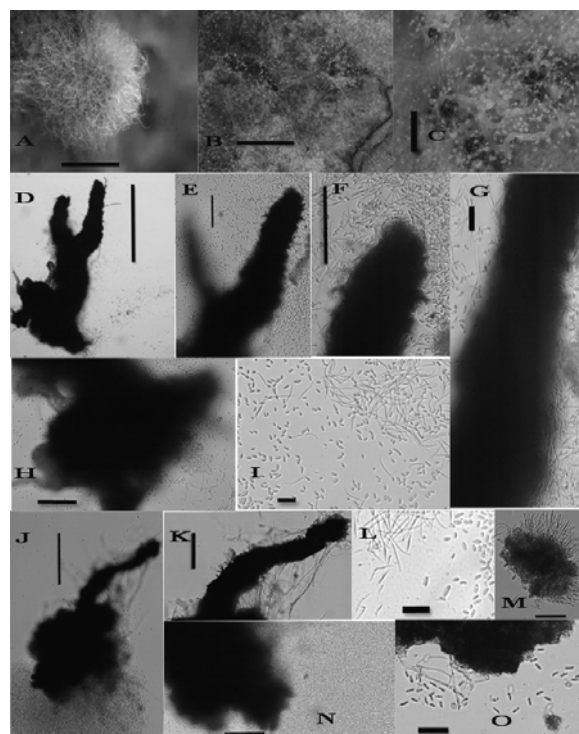

Fig. 2 Morphological characteristics of pycnidia grown on leaves of *Mangifera indica* after 60d. A, B: beakless and long-necked pycnidia, picture A was taken after 7d cultivation; C: exudates of pycnidia; D-H: different parts of branched pycnidia; I, L, O: α- and ß-conidia; J, K, and N: different parts of long-necked pycnidia; M: conidiophores. Bars A-C, 1000 μm; D, J: 500 μm; E-H, K, N: 100 μm; I, L, O: 20 μm; M: 40 μm.
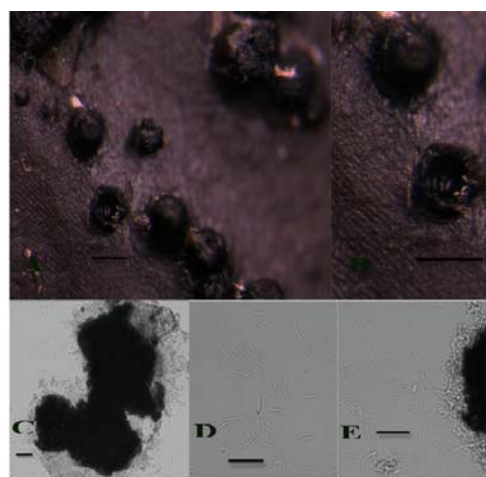

Fig. 3 Morphological characteristics of pycnidia grown on leaves of *Kandelia candel* after 60d. A, B: Scattered and irregular pycnidia, picture B was part of A; C: Crushed pycnidia taken from that of in picture B. D-E: ß-conidia. Bars A-B, 1000 μm; C-E: 40μm.

Phylogenetic analysis

Two alignments were analyzed, the first alignment consisted of 34 *Phomopsis/Diaporthe* taxa and two outgroup taxa from the

genera *Leucostoma* (AF191170, AF191175), related genera in the *Diaporthales*, downloaded from the GenBank. The alignment included 632 total characters, of which ambiguously aligned were excluded. The evolutionary trees constructed by the maximum likelihood and distance trees estimated by the neighbor joining analysis were similar in their topologies and were com-

pletely congruent with the phylogeny inferred by the Bayesian analysis (Fig. 4). The group of *D. phaseolorum* containing mangrove endophytic fungus 1893, together with other isolates from different host genera, was supported with high values (100 and 1.00 for NJ and Bayes analysis, respectively), which suggested our endophyte be a member of *D. phaseolorum*.
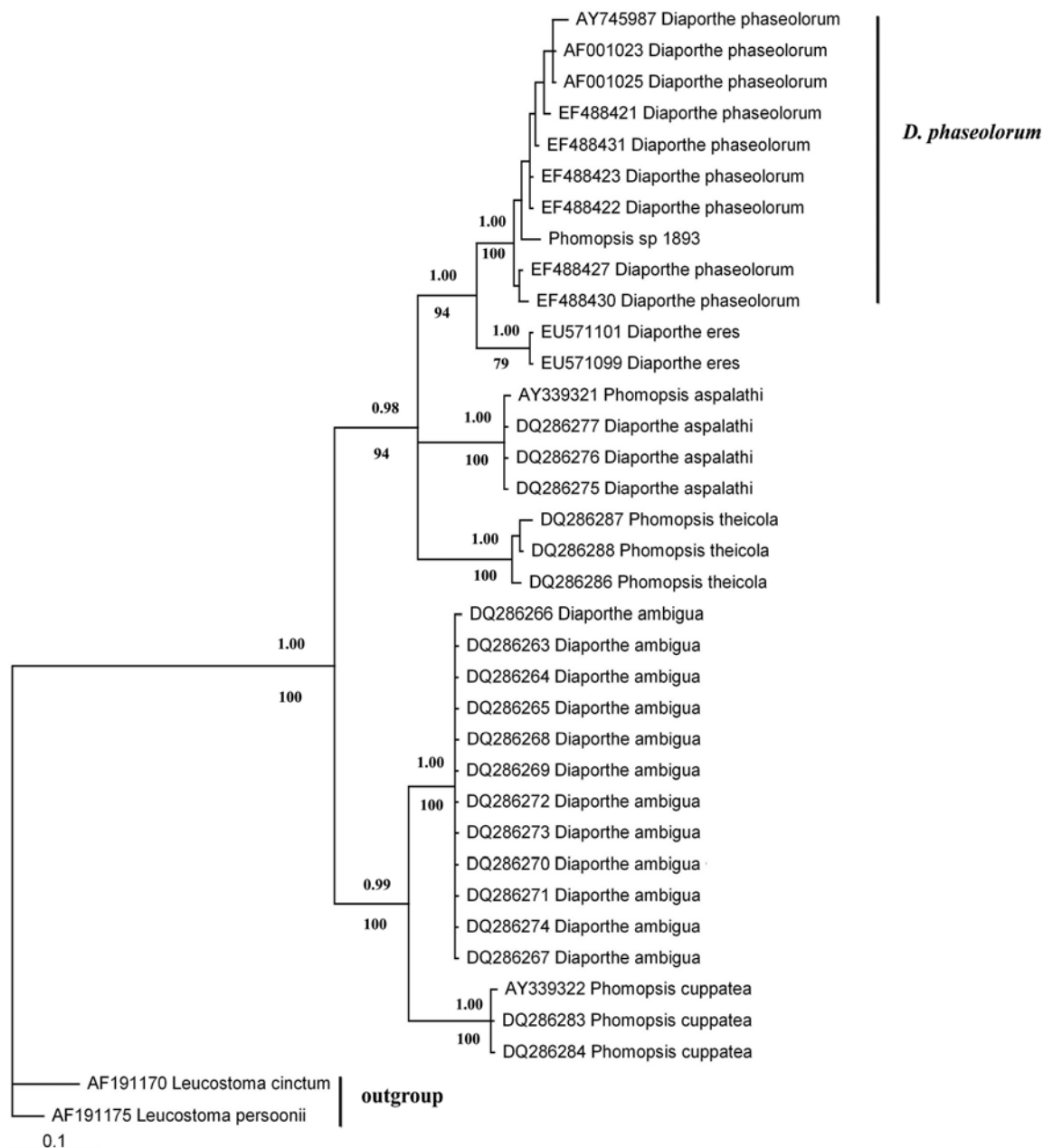

Fig. 4 Phylogenetic analysis of mangrove endophytic fungus 1893 inferred from ITS. The majority rule consensus tree derived by maximum likelihood analysis. Bayesian posterior probabilities and NJ-bootstrapping values (>50%) are shown above and below the lines, respectively. *Leucostoma cinctum* (AF191170) and *Leucostoma persoonii* (AF191175) were used as outgroup.

The second alignment consisted only of sequences of *D. phaseolorum* and three other *D. phaseolorum* varieties (*D. phaseolorum* var. *sojae*, var. *caulivora*, and var. *meridionalis*)

without rooting (Figure 5). This alignment included 597 total characters of which ambiguously aligned were excluded. Analysis of the entire sequences based on equally weight character

states by maximum likelihood, Neighbor-joining and Bayesian analysis resulted in clustering the endophyte in the clade of *D. phaseolorum* var. *sojae* (AF001017, AF001018, AF001021,

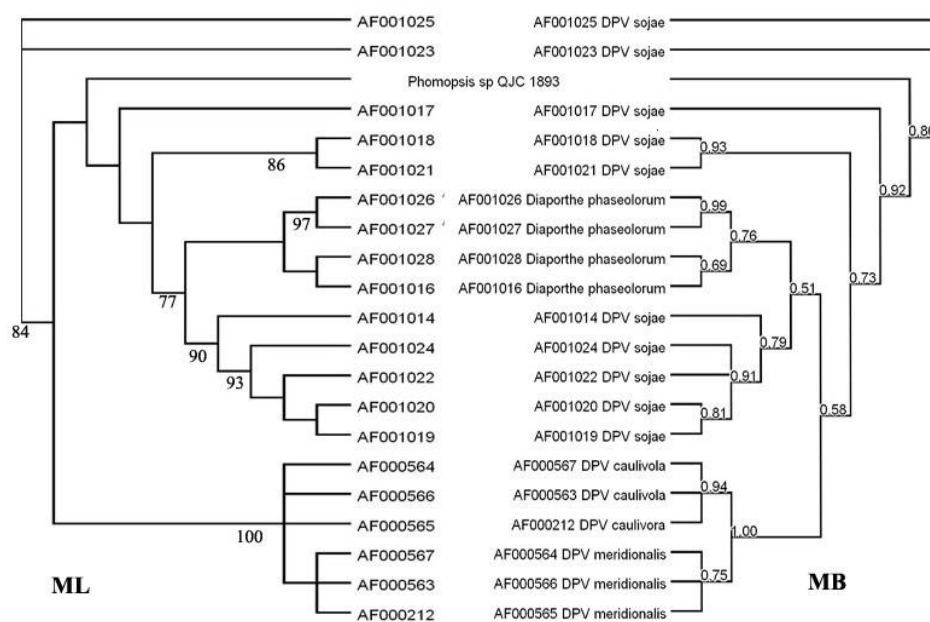AF001023 and AF001025). Finally, the endophyte was identified as *D. phaseolorum* var. *sojae*.

Fig. 5   **Phylogenetic analysis of mangrove endophytic fungus 1893 (*Phomopsis* sp QJC 1893) with other *D. phaseolorum* varieties inferred from ITS.** The majority rule consensus tree (part MB) was derived by Bayesian analysis. Bayesian posterior probabilities values (>50%) were shown above the lines. One of tree derived by maximum likelihood analysis was shown in part ML with NJ-bootstrapping values (>50%) shown below the line.

Discussion

Phomopsis (Sacc.) Bubák is a large, coelomycetous genus that includes over 1000 species names described primarily on the basis of plant host (Rossman et al. 2007; Van Rensburg et al. 2006), which is also the most prevalent endophytic fungus isolated from both tropical and temperate woody plants (Murali et al. 2006; Rossman et al. 2007) Recently, inconsistencies of association within monophyletic groups based on ITS sequences and morphological systematics have been noted and genetic diversity within *Phomopsis* isolates from divergent plant hosts evaluated by cladistic analyses using ITS sequences (Kanematsu et al. 2000) resulted that the host-based species concept in the genus *Phomopsis* was not reliable (Mostert et al. 2001). Studies by Rehner and Uecker (1994), and Zhang et al (1998) suggested that delimiting species within the genus *Phomopsis* is more complex than previously recognized. Using ITS sequences, *P. amygdali* has recently been identified from cultivated grape, a non-rosaceous host (Mostert et al. 2001). According to Farr et al. (1999) some species of *Phomopsis* appear to be restricted to one plant host genus or family, while other species can be isolated from diverse plant hosts. Conversely, strains of *Phomopsis* isolated from one host species are not necessarily closely related and may represent more than one taxon. In a study of *Diaporthe/Phomopsis* complex on soybean (*P. longicolla*, *D. phaseolorum* var. *sojae*, var. *caulivora*, and var. *meridianalis*) was inferred based on ITS sequences. Though the correct usage of names (i.e., species, forma

specialis and varieties) is still an issue (Hobbs et al. 1985), var. *caulivora* and var. *meridionalis* formed different, well-supported monophyletic groups and var. *sojae* were phylogenetically divergent with a wider host range and more variable in morphological characteristics (Kulik 1984). In our study, mangrove endophytic fungus 1893 was classified as *D. phaseolorum* var. *sojae* based on morphological and molecular evidence. This is the first report of *D. phaseolorum* var. *sojae* isolated from *Kandelia candel*. Our result also suggests *D. phaseolorum* var. *sojae* be either a variety of *D. phaseolorum* or possible a distinct species with widespread host rang.

Acknowledgements

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